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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07D 295/02, A61K 31/505 — A1

(11) International Publication Number: WO 97/33879

(43) International Publication Date: 18 September 1997 (18.09.97)

(21) International Application Number: PCT/US97/03648

(22) International Filing Date: 11 March 1997 (11.03.97)

(30) Priority Data:

| | | |
|---|---|---|
| 60/013,485 | 15 March 1996 (15.03.96) | US |
| 9610365.0 | 17 May 1996 (17.05.96) | GB |

(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
*With international search report.*

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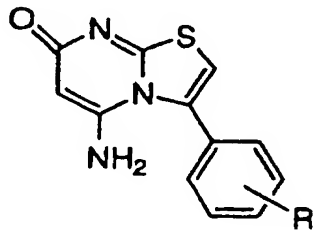
(54) Title: COMPOUNDS AND METHODS FOR SELECTIVELY INHIBITING ACTIVATION OF THE HUMAN A3 ADENOSINE RECEPTOR

(57) Abstract

The invention is a method for inhibiting activation of the human A3 adenosine receptor with adenosine, by treating the receptor with a compound of formula (I); e.g., (II).

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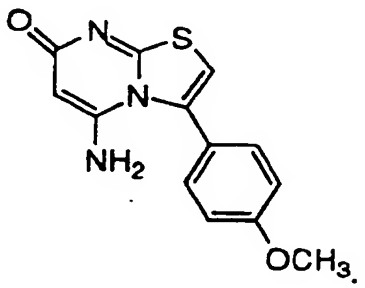


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TITLE OF THE INVENTION

# COMPOUNDS AND METHODS FOR SELECTIVELY INHIBITING ACTIVATION OF THE HUMAN A3 ADENOSINE RECEPTOR

## BACKGROUND OF THE INVENTION

The present invention concerns the use of compounds as selective antagonists of the A3 adenosine receptor subtype for preventing mast cell degranulation and are therefore useful in the treatment or prevention of disease states induced by activation of the A3 receptor and mast cell activation. These disease states include but are not limited to asthma, myocardial reperfusion injury, allergic reactions including but not limited to rhinitis, poison ivy induced responses, urticaria, scleroderma, arthritis, other autoimmune diseases and inflammatory bowel diseases. The selective antagonists are selective for the A3 adenosine receptor over the A1, A2a, and A2b adenosine receptors.

The actions of adenosine are mediated through G-protein coupled receptors, the A1, A2a, A2b and A3 adenosine receptors. The adenosine receptors were initially classified into A1 and A2 subtypes on the basis of pharmacological criteria and coupling to adenylate cyclase (Van Caulker, D., Muller, M. and Hamprecht, B. (1979) *J. Neurochem.*, *33*:999-1003). Further pharmacological classification of adenosine receptors prompted subdivision of the A2 class into A2a and A2b subtypes on the basis of high and low affinity, respectively, for adenosine and the agonists NECA and CGS-21680 (Bruns, R.F., Lu, G.H. and Pugsley, T.A. (1986) *Mol. Pharmacol.*, *29*:331-346; Wan, W., Sutherland, G.R. and Geiger, J.D. (1990) *J. Neurochem.*, *55*:1763-1771). Molecular cloning and characterization of the human A3 adenosine receptor is described in Salvatore *et al.*, *Proc. Natl. Acad. Sci. USA*, vol. (90) pp 10365-10369, November 1993. The existence of A1, A2a, A2b and A3 subtypes has been confirmed by cloning and functional characterization of expressed bovine, canine, rat and human receptors. Cloning and characterization of the human A1, A2a, A2b and A3 receptors are described in GB 2264948-A. Based on the use of these cloned receptors, an assay has been described to identify adenosine

receptor agonists and antagonists and determine their binding affinity (see GB 2 264 948 A, published 9/15/93; see also R.F. Bruns *et al.*, (1983) *Proc. Natl. Acad. Sci. USA*, *80*:2077-2080; R.F. Bruns *et al.*,(1986) *Mol. Pharmacol.*, *29*:331-346; M.F. Jarvis *et al.*, (1989) *J. Pharma. Exp. Therap.*, *251*:888-893; K.A. Jacobson *et al.*, (1989) *J. Med. Chem.*, *32*:1043-1051).

Adenosine exhibits diverse and potent physiological actions in the cardiovascular, nervous, pulmonary, renal and immune systems. Adenosine has been demonstrated to terminate superventricular tachycardia through blockage of atrioventricular nodal conduction (J.P. DiMarco *et al.*, (1985) *J. Am. Col. Cardiol.*, *6*:417-425, A. Munoz *et al.*, (1984) *Eur. Heart J.*, *5*:735-738). Adenosine is a potent vasodilator except in the kidney and placenta (R.A. Olsson, (1981) *Ann. Rev. Physiol.*, *43*:385-395). Adenosine produces bronchoconstriction in asthmatics but not in nonasthmatics (Cushly *et al.*, 1984, *Am. Rev. Respir. Dis.*, *129*:380-384). Adenosine has been implicated as a preventative agent and in treatment of ventricular dysfunction following episodes of regional or global ischemia (M.B. Forman and C.E. Velasco (1991) *Cardiovasc. Drugs and Therapy*, *5*:901-908) and in cerebral ischemia (M.C. Evans *et al.*, (1987) *Neurosci. Lett.*, *83*:287, D.K.J.E.,Von Lubitz *et al.*, (1988) *Stroke*, *19*:1133).

Adenosine receptor agonists, antagonists and binding enhancers have been identified and implicated for usage in the treatment of physiological complications resulting from cardiovascular, pulmonary, renal and neurological disorders. Adenosine receptor agonists have been identified for use as vasodilators ((1989) *FASEB. J.*, *3*(4) Abs 4770 and 4773, (19910 *J. Med. Chem.*, (1988) *34*:2570), antihypertensive agents (D.G. Taylor *et al.*, *FASEB J.*, (1988) 2:1799), and anti-psychotic agents (T.G. Heffner *et al.*, (1989) *Psychopharmacology*, *98*:31-38). Adenosine receptor agonists have been identified for use in improving renal function (R.D. Murray and P.C. Churchill,(1985) *J. Pharmacol. Exp. Therap.*, *232*:189-193). Adenosine receptor allosteric or binding enhancers have shown utility in the treatment of ischemia, seizures or hypoxia of the

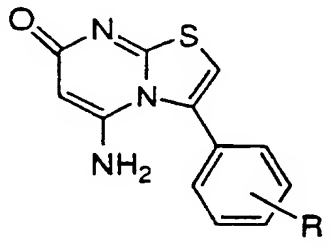
brain (R.F. Bruns, *et al.* (1990) *Mol. Pharmacol., 38*:939-949; C.A. Janusz, *et al.*, (1991) *Brain Research, 567*:181-187).

Methods of treating conditions related to the physiological action of adenosine have, to date, proven inferior due to the nonselectivity of the compounds for the multiple adenosine receptor subtypes present in whole tissue (R.F. Bruns *et al.*, (1986) *Mol. Pharm., 29*:331-346) and the inability to extrapolate activities measured on non-human tissues due to the species variability in the affinity for adenosine analogs and the physiological effects of adenosine (Ukera, *et al.*, (1986) *FEBS Lett, 209*:122-128, Stone, *et al.*(1988) 15, 31-46).

We have identified compounds which selectively inhibit activation of the human adenosine A3 receptor over the A2a and A2b receptors, and therefore provide a method of using such compounds which overcomes the disadvantages of using compounds of uncharacterized specificity.

## SUMMARY OF THE INVENTION

The invention concerns new methods for inhibiting activation of the human A3 adenosine receptor by adenosine, by treating a patient in need thereof with a compound of the formula




I

and pharmaceutically acceptable salts thereof, wherein R is selected from the group consisting of

hydrogen,
chlorine,
bromine,

fluorine,
iodine,
hydroxyl,
C1-4 alkyl,
C1-4 alkyloxy, and
C1-4 alkylcarboxy,

referred to hereinafter as A3 adenosine receptor antagonists.

The invention includes a method for achieving blockade of the vasoconstrictive response induced through adenosine activation of the A3 adenosine receptor subtype, which comprises treating the patient with an A3 adenosine receptor antagonist described above.

The invention also includes a method for treating or preventing myocardial ischemia, inflammation, brain arteriole diameter constriction, and the release of allergic mediators, which comprises treating the patient with an A3 adenosine receptor antagonist described above.

The invention also includes a method for treating or preventing asthma, myocardial reperfusion injury, rhinitis, poison ivy induced responses, urticaria, scleroderma, arthritis, and inflammatory bowel diseases which comprises treating the patient with an A3 adenosine receptor antagonist described above.

The invention also includes a method for preventing mast cell degranulation in a human which comprises treating the patient with an A3 adenosine receptor antagonist described above.

## DETAILED DESCRIPTION OF THE INVENTION

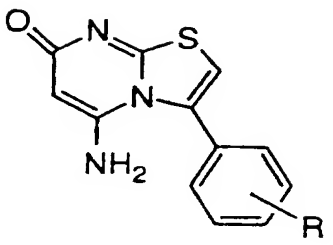
Adenosine, adenosine metabolites and other A3 adenosine receptor agonists induce mast cell degranulation. Mast cell activation promotes the release of enzymes, bioactive amines and arachidonic acid metabolites which causes vasoconstriction, edema, leukocyte accumulation, and ultimately, tissue damage. Mast cell degranulation is associated with myocardial reperfusion injury, hypersensitivity reactions such as asthma, allergic rhinitis, urticaria, ischemic bowel disease,

autoimmune diseases including autoimmune inflammation, and atopic dermatitis.

The invention consists of administering an inhibitory effective amount of the A3 adenosine receptor antagonist to treat or prevent these diseases and pathologic effects that result from mast cell degranulation. The types of diseases amenable to treatment by the method of this invention include, but are not limited to, human diseases such as Addison's disease, autoimmune hemolytic anemia, Crohn's disease, Goodpasture's syndrome, Grave's disease, Hashimoto's thyroiditis, idiopathic thrombocytopinic purpura, Insulin-dependent diabetes militus, multiple sclerosis, myasthenia gravis, Pemphigus vulgaris, pernicious anemia, poststreptococcal glomerulonephritis, psoriasis, rheumatoid arthritis, scleroderma, Sjogren's syndrome, spontaneous infertility, and systemic lupus erythematosus, and animal skin disorders and allergies.

In one embodiment of the invention, the method provides a means for preventing or treating disease states associated with vascular constriction induced through activation of the A3 subtype of the adenosine receptor. The method comprises contacting said receptor in the vasculature with an amount of A3 adenosine receptor antagonist which selectively blocks activation of the A3 adenosine receptor subtype on granulocytes, including mast cells, exhibiting the A3 adenosine receptor. A3 adenosine receptor antagonists are used to effect a reduction in vasoconstriction in the vasculature without any substantial effect (binding or blockade) of the A2a or A2b subtypes of the adenosine receptor.

The methods involve inhibiting activation of the human A3 adenosine receptor by adenosine, by treating the patient in need thereof with a compound of the formula



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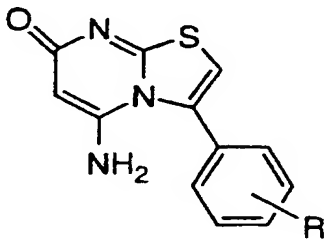
and pharmaceutically acceptable salts thereof, wherein R is selected from the group consisting of

hydrogen,
chlorine,
bromine,
fluorine,
iodine,
hydroxyl,
$C_{1-4}$ alkyl,
$C_{1-4}$ alkyloxy, and
$C_{1-4}$ alkylcarboxy.

In one class of compounds used in the method, R is hydrogen, $C_{1-4}$ alkyl or $C_{1-4}$ alkyloxy. In one embodiment of this class, R is $-OCH_3$.

The invention also includes compounds having the formula



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and pharmaceutically acceptable salts thereof, wherein R is selected from the group consisting of

fluorine,
iodine,
$C_{2-4}$ alkyl,
$C_{3-4}$ alkyloxy, and
$C_{1-4}$ alkylcarboxy.

The invention also includes use of a compound of the invention or a pharmaceutically acceptable salt thereof, in the manufacture of a medicament for treating or preventing asthma, myocardial reperfusion injury, rhinitis, poison ivy induced responses, urticaria, scleroderma, arthritis, and inflammatory bowel diseases in a mammal.

The term "alkyl" means straight or branched alkane containing 1 to about 4 carbon atoms, e.g., methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, and tert-butyl.

The term "alkyloxy" or includes an alkyl portion where alkyl is as defined above, e.g., methyloxy, propyloxy, and butyloxy.

The term "alkylcarboxy" includes an alkyl portion where alkyl is as defined above, e.g., methylcarboxy, propylcarboxy, and butylcarboxy.

The term "oxy" means an oxygen (O) atom.

The term "pharmaceutically acceptable salts" shall mean non-toxic salts of the compounds of this invention which are generally prepared by reacting the free base with a suitable organic or inorganic acid. Representative salts include the following salts: acetate, benzenesulfonate, benzoate, bicarbonate, bisulfate, bitartrate, borate, bromide, calcium edetate, camsylate, carbonate, chloride, clavulanate, citrate, dihydrochloride, edetate, edisylate, estolate, esylate, fumarate, gluceptate, gluconate, glutamate, glycollylarsanilate, hexylresorcinate, hydrabamine, hydrobromide, hydrochloride, hydroxynapthoate, iodide, isothionate, lactate, lactobionate, laurate, malate, maleate, mandelate, mesylate, methylbromide, methylnitrate, methylsulfate, mucate, napsylate, nitrate, oleate, oxalate, pamaote, palmitate, panthothenate, phosphate/diphosphate, polygalacturonate, salicylate, stearate, subacetate, succinate, tannate, tartrate, teoclate, tosylate, triethiodide, valerate.

A3 adenosine receptor antagonists useful for the present invention can be prepared according to the general procedure exemplified in El-Kerdawy, Synthesis of Novel Thiazolopyrimidine and Thiazolothiatriazine Derivatives; *Bull. Fac. Cairo Univ.*,: Vol 31. No. 1. (1993). According to the general procedure for producing 3-Aryl-5-amino-7-oxo-thiazolo[3,2]-pyrimidines, a solution of the appropriate 2-amino-4-arylthiazole (e.g., 2-amino-4-phenylthiazole (Aldrich), 2-amino-4-(4-chlorophenyl)thiazole (Aldrich)) in acetic acid is heated under reflux with ethyl cyanaoacetate for several hours. The cooled reaction mixture is treated with ice water and neutralized with ammonium hydroxide. The precipitate formed is collected, washed with water, dried and crystallized from aqueous methanol.

## EXAMPLE 1

Synthesis of 3-(4-methoxyphenyl)-5-amino-7-oxo-thiazolo[3,2]-pyrimidine (1-1)

A solution of the 2-amino-4-(4-methoxyphenyl)-thiazole (0.01 mole) (Menai Organics Ltd., Gwynedd, United Kingdom) in acetic acid (10 ml) was heated under with ethyl cyanaoacetate (1.13 g, 0.01 mole) for 6 hrs. The cooled reaction mixture was treated with ice water and neutralized with 10% ammonium hydroxide. The precipitate formed was collected, washed with water, dried and crystallized from aqueous methanol.

MP = 185-186°C
MF = $C_{13}H_{11}N_3O_2S$
MW = 273.31
Calc. C 57.13%, H 4.06%, N 11.73%
Found C 57.3%, H 4.2%, N 11.5%

The A3 adenosine receptor antagonists useful for the present invention can be administered in such oral forms as tablets, capsules (each of which includes sustained release or timed release formulations), pills, powders, granules, elixirs, tinctures, suspensions, syrups, and

emulsions. Likewise, they may be administered in intravenous (bolus or infusion), intraperitoneal, subcutaneous, or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts.

The dosage regimen utilizing the compounds of the present invention is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal and hepatic function of the patient; and the particular compound or salt thereof employed. An ordinarily skilled physician or veterinarian can readily determine and prescribe the effective amount of the A3 adenosine receptor antagonist required to prevent, counter, or arrest the progress of the condition.

Oral dosages of the A3 adenosine receptor antagonists, when used for the indicated effects, will range between about 1 µg per kg of body weight (µg/kg) to about 10 mg/kg intravenously, e.g., 10 µg/kg, 1 mg/kg, or 5 mg/kg. For a typical patient, doses range from between 0.1 mg to 1 gram, e.g., 1 mg, 100 mg and 500 mg. Advantageously, A3 adenosine receptor antagonists useful for the present invention may be administered in divided doses of two, three, or four times daily.

Intravenously, the most preferred doses of A3 adenosine receptor antagonist will range from about 0.05 to about 0.25 µg/kg/minute during a constant rate infusion, e.g., 0.15 µg/kg/minute. In order to administer that amount of active ingredient, a composition of the invention having 0.05 mg/ml of active ingredient should be administered at a rate of between about 0.001 and 0.005 ml/kg/min, e.g., 0.003 ml/kg/min. Compositions of the invention containing higher concentrations of active ingredients should be administered at correspondingly lower rates.

Furthermore, preferred A3 adenosine receptor antagonists useful for the present invention can be administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in that art. To be administered in the form of a transdermal delivery

system, the dosage administration will, of course, be continuous rather that intermittent throughout the dosage regime.

In the methods of the present invention, the A3 adenosine receptor antagonists herein described form the active ingredient, and are typically administered in admixture with suitable pharmaceutical diluents, excipients or carriers (collectively referred to herein as "carrier" materials) suitably selected with respect to the intended form of administration, that is, oral tablets, capsules, elixirs, syrups and the like, and consistent with convention pharmaceutical practices.

For instance, for oral administration in the form of a tablet or capsule, the A3 adenosine receptor antagonist component can be combined with an oral, non-toxic, pharmaceutically acceptable, inert carrier such as lactose, starch, sucrose, glucose, methyl cellulose, magnesium stearate, dicalcium phosphate, calcium sulfate, mannitol, sorbitol and the like; for oral administration in liquid form, the oral drug components can be combined with any oral, non-toxic, pharmaceutically acceptable inert carrier such as ethanol, glycerol, water and the like. Moreover, when desired or necessary, suitable binders, lubricants, distintegrating agents and coloring agents can also be incorporated into the mixture. Suitable binders include starch, gelatin, natural sugars such as glucose or beta-lactose, corn-sweeteners, natural and synthetic gums such as acacia, tragacanth or sodium alginate, carboxymethylcellulose, polyethylene glycol, waxes and the like. Lubricants used in these dosage forms include sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chloride and the like. Disintegrators include, without limitation, starch, methyl cellulose, agar, bentonite, xanthan gum and the like.

The A3 adenosine receptor antagonists useful for the present invention can also be administered in the form of liposome delivery systems, such as small unilamellar vesicles, large unilamellar vesicles and multilamellar vesicles. Liposomes can be formed from a variety of phospholipids, such as cholesterol, stearylamine or phosphatidylcholines.

A3 adenosine receptor antagonists useful for the present invention may also be delivered by the use of monoclonal antibodies as

individual carriers to which the compound molecules are coupled. The compounds of the present invention may also be coupled with soluble polymers as targetable drug carriers. Such polymers can include polyvinylpyrrolidone, pyran copolymer, polyhydroxy-propyl-methacrylamide-phenol, polyhydroxy-ethyl-aspartamide-phenol, or polyethyleneoxide-polylysine substituted with palmitoyl residues. Furthermore, the compounds of the present invention may be coupled to a class of biodegradable polymers useful in achieving controlled release of a drug, for example, polylactic acid, polyglycolic acid, copolymers of polylactic and polyglycolic acid, polyepsilon caprolactone, polyhydroxy butyric acid, polyorthoesters, polyacetals, polydihydropyrans, polycyanoacrylates and cross linked or amphipathic block copolymers of hydrogels.

Adenosine has been shown to produce bronchoconstriction in asthmatics but not in nonasthmatics, demonstrating that adenosine plays a role in the etiology of this disease state (Cushly *et al.*, *AM. Rev. Respir. Dis.*, (129) pp. 380-384 (1984)). Adenosine mediated bronchoconstriction on bronchial rings in asthmatics is blocked *in vitro* by a combination of histamine and leukotriene antagonists (Bjorck *et al.*, *Am. Rev. Resp. Dis.*, 1992, (145) pp. 1087-1091 (1992)), indicating that adenosine acts by releasing histamine, leukotriene and other agents from mast cells or other cells that contain these allergic mediators. A3 adenosine receptor antagonists identified herein as being useful to antagonize the A3 adenosine receptor can be used in conjunction with other therapies, including co-administration of anti-histamine, leukotriene blockade or other anti-allergic mediator therapies and A3 specific antagonists.

Blockade of A3 adenosine receptor mediated action in the vasculature is useful to treat and prevent disease states in humans. Adenosine potentiates the release of granule contents from mast cells isolated from rat peritoneum (see Lohse *et al.*, *N.-S. Arch. Pharmacol.*, (335) pp. 555-560 (1987) and Marquardt *et al.*, *J. Immunol.*, (120) pp. 871-878 (1978)), which causes constriction in some vascular beds resulting in C5a-induced myocardial ischemia (see Ito *et al.*, *Am. J.*

*Physiol.*, (264) (*Heart Circ. Physiol.*, (33) pp. H1346-H1354 (1993)), mast cell-dependent inflammation (see Raud, J., *Acta. Physiol. Scand.*, (135) (Suppl., 578) pp. 1-58 (1989)), brain arteriole diameter constriction (see Rosenblum, W. I., *Brain Res.*, (49) pp. 75-82 (1973)), and the release of allergic mediators (see Ramkumar, *et al.*, *J. Biol. Chem.*, (268) pp. 16887-16890 (1993)). These disease states can be prevented or treated by contacting A3 receptor bearing mast cells with an amount of an A3 adenosine receptor antagonist identified herein effective to prevent mast cell degranulation.

The trigger for mast cell degranulation is usually thought to be an allergen. Allergens are endocytosed by marcrophages and degraded. The resulting fragments are displayed on T lymphocytes. B lymphocytes are stimulated to mature into plasma cells which are able to secrete allergen-specific molecules known as immunoglobulin E. These antibodies attach to receptors on mast cells in tissue and on basophils circulating in blood to trigger degranulation (see L. Lichtenstein, *Sci. Am.*, (269) pp. 116-125 (1993)). Activation of A3 adenosine receptors can produce mast cell degranulation and enhance the effect of allergens. Adenosine and antigens trigger an influx of calcium to induce mast cell granules to release their contents an promote synthesis and release of cytokines, prostaglandins and leukotrienes. The various chemicals released by mast cells are responsible for many allergic symptoms. Long term release of these chemicals can induce basophils, eosinophils, and other cells flowing through blood vessels to migrate into the tissue. Migration is promoted due to the expression and activation of adhesion molecules on the circulating cells and on vascular endotheilial cells. The circulating cells adhere to the endothelial cells, roll among them, and eventually cross into the surrounding matrix. These recruited cells secrete chemicals of their own that damage tissue. Thus, there are long term secondary effects which may also be prevented by specific blockade of mast cell degranulation.

The A3 adenosine receptor antagonists are useful for treating patients prone to reperfusion injury, including those with coronary artery diseases in general, and patients anticipating the opening of occluded

arteries (reperfusion) by various interventions, e.g., coronary artery bypass grafts, angioplasty or thrombolytic therapy. Heller, L.J. and Regal, J.F., "Effect of adenosine on histamine release and atrioventricular conduction during guinea pig cardia anaphylaxis" *Circ. Res.*, (62) pp. 1147-1158 (1988), suggest that mast cell degranulation is involved in ischemia/reperfusion injury. Adenosine-induced mast cell degranulation during a period of transient ischemia may be responsible for the phenomenon of preconditioning (i.e., a transient ischemic episode reduces myocardial damage resulting from a subsequent prolonged ischemic episode). Accordingly, mast cells are temporarily depleted of damaging mediators during the preconditioning period. Heller *et al.* found increases in levels of endogenous adenosine during cardiac anaphylaxis contributed to the development of atrioventricular conduction delays and that increases in levels of adenosine before antigen challenge may increase the amount of histamine released during cardiac anaphylactic reactions. Wolff *et al.*, "Ventricular arrhythmias parallel cardiac histamine efflex after coronary artery occlusion in the dog" *Agents and Actions*, (25) pp. 296-306 (1988), concluded that during acute myocardial ischemia, the coronary sinus histamine concentration increases simultaneously with the development of early ischemic ventricular arrhythmias and in proportion of their severity. Keller *et al.*, "Acute reoxygenation injury in the isolated rat heart: role of resident cardiac mast cells" *Circ. Res.*, (63) pp. 1044-1052 (1988), found that isolated crystalloid-perfused rat heart is not a leukocyte-free preparation and mast cells resident to the heart play an important role in acute reoxygenation injury. Jolly *et al.*, "Effects of lodoxamide on ischemic reperfused myocardium" *J. Cardiovas. Pharmacol.*, (4) pp. 441-448 (1982), found that lodoxamide, a drug that acts to inhibit mast cell degranulation, reduces myocardial ischemic injury. Ito *et al.*, "Role of cardiac mast cells in complement C5a-induced myocardial ischemia" *Am. J. Physiol.*, (33) pp. H1346-H1354 (1992), found that cardiac mast cells are involved in complement-induced release of vasoactive eicosanoids, including TxA2.

For example, the A3 receptor antagonists can be used in adjunct therapy with recanulation such as angioplasty and thrombolytic agents for the treatment of reperfusion injury. A probable course of treatment would be acute i.v. formulation followed by at least two weeks oral treatment for those patients undergoing angioplasty or thrombolytic therapy with, for example, TPA or steptokinase. For those patients presenting with angina, chronic oral therapy would be appropriate.

Radioligand Binding Assay

The human A2a, A2b and A3 receptor subtype cDNAs were subcloned into the expression vectors pSVL (Pharmacia, Columbus, Ohio), CMV5 (Mumby *et al.*, *PNAS*, (87) pp. 728-732 (1990)) pCDNA1 or pREP (Invitrogen). Transient expression in COS7 cells (monkey kidney cell line, ATCC CRL 1651, ATCC, Rockville, MD) (Doyl *et al.* WO 95/11681) was accomplished by transfection of the cloned adenosine receptor cDNAs under the control of the SV40 promoter into mammalian cells, such as COS7. Membranes prepared from the transfected cells were utilized for the determination of binding affinity, selectivity and specificity of the human adenosine receptors for various ligands. Stable expression of the human adenosine receptors in mammalian cells (e.g., CHO, HEK 293) was achieved after integration of the transfected cDNA into the chromosomes of the host cells. These stable cell lines constituently express the cloned human adenosine receptors and can be propagated infinitely. Stable cell lines expressing the human adenosine subtype cDNAs individually can be used in the binding assay to measure the affinity and selectivity of the receptors for adenosine agonists, antagonists and enhancers.

Membranes prepared from transfected COS7 and CHO cells were utilized in a binding assay to measure the affinity of agonists and antagonists on the human adenosine receptors. Monolayer cell culture of transfected COS7 cells were dissociated with 1 mM EDTA in phosphate buffered saline and resuspended in 5 mM Tris, pH 7.6/10 mM $MgCl_2$. The cells were subjected to freeze-thaw lysis and the suspension was homogenized in a glass dounce homogenizer. The membranes were

pelleted, resuspended in binding buffer, 50 mM Tris pH 7.6/10 mM $MgCl_2$ and incubated with adenosine deaminase before the binding assay. The binding assay was performed by incubating 50-100 µg of membranes with increasing concentrations of radiolabeled adenosine agonists. Bound ligand was separated from free ligand by filtration on a Skatron cell harvester equipped with a receptor binding filtermat. Bound radioactivity was measured by scintillation counting. Binding data were analyzed by the use on nonlinear regression curve fitting program Graph Pad InPlot, Version 3.0 (Graph Pad Software, San Diego). $K_i$ values were calculated using the Cheng-Prusoff derivation (Cheng, Y.C. and Prusoff, H.R. (1973) *Biochem. Pharmacol.* 22, 3099-3108). The affinities of agonists and antagonists on human adenosine receptor subtypes were measured in competition binding assays using the radiolabeled adenosine agonists [$^3$H]-CGS21680 (2-(p-(2-carboxyethyl)-phenylamino)-5'-N-ethyl-carboxamidoadenosine) for A2a receptors and, [$^3$H]-5'-N-ethylcarboxamido adenosine ([$^3$H]-NECA), or [$^{125}$I]-N6-aminobenzyl adenosine ($^{125}$I-ABA) for $A_3$ receptors. Nonselective binding was determined with 1µM I-ABA for the $A_3$ receptor and 10 µM NECA for $A_{2a}$ receptors. Activity on $A_{2b}$ receptor subtypes was measured in the cAMP accumulation assay described below.

cAMP accumulation assay

The changes in cAMP accumulation were measured in stably transfected CHO cells expressing the human adenosine receptor subtypes. CHO cells were washed twice in phosphate buffered saline (PBS) and detached in 0.2% EDTA in PBS. After pelleting at 800 rpm for 10 min, cells were resuspended in KRH buffer (140 mM NaCl/5 mM KCl/2 mM $CaCl_2$/1.2 mM $MgSO_4$/1.2 mM $KH_2PO_4$/6 mM glucose/25 mM Hepes buffer, pH 7.4), washed once in KRH buffer and resuspended at $10^7$ cells/mL. The cell suspension (100 µL) was mixed with 100 µL of KRH buffer containing 200 µM of the phosphodiesterase inhibitor Ro 20-1724 and incubated at 37°C for 15 minutes. Antagonists were preincubated for 10 minutes before adding 5 µM forskolin and 60 µM adenosine. After 10 minutes, 400 µL of 100 mM acetic acid was added and the sample was

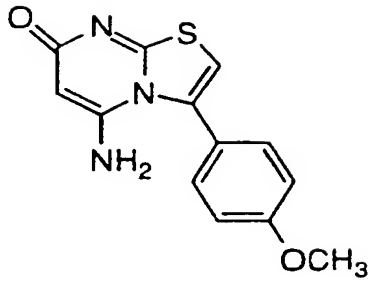
boiled for 5 minutes. The supernatant was recovered by centrifugation for 15 minutes and cAMP levels were determined by radioimmunoassay (RIANEN kit, DuPont/NEN) using the acetylation protocol. For measurement on $A_{2b}$ receptors, the increase in cAMP accumulation was induced by addition of adenosine (10 μM) and incubated at 37°C for 20 minutes before termination with acidic acid and RIA analysis. The ability to block the agonist-induced increase in cAMP was measured by preincubating antagonists for 10 minutes before adding 10 μM adenosine.

In determining the quantities of antagonist necessary to block adenosine binding to the A3 receptor, persons skilled in the art would recognize that an A3 adenosine receptor antagonist with high affinity for the receptor can be administered at dosages lower than A3 adenosine receptor antagonists with low affinity. A3 adenosine receptor antagonists having a pKi of greater than about 7 for the A3 receptor and below about 6 for other adenosine receptor subtypes, may be administered by any effective means to achieve either localized or systemic contact of the A3 adenosine receptor antagonist with target A3 adenosine receptors, including intravenous, intramuscular, intrasynovial, intranasal, nebulized intrapulmanory, intraperitoneal or other common means for administration of therapeutic compounds. Dosages of between about 1 μg/kg and 10 mg/kg are envisioned, as necessary, to achieve the desired effect of A3 adenosine receptor blockade.

The following examples are provided to further define but not to limit the invention defined by the foregoing description and the claims which follow:

Selectivity for the A3 adenosine receptor

$IC_{50}$ values, representing the amount of



required to inhibit specific binding of the radioligand to the A3 receptor subtype by 50%, and cAMP accumulation via A2b receptor subtype were determined. Activity on A2a and A2b receptor subtypes was measured at a single concentration of antagonist (10 μM) and is reported as % inhibition of specific binding.

| Receptor | % Inhibition at 10 μM |
|---|---|
| A2a | 33% |
| A2b | 34% |

| Receptor | $IC_{50}$ |
|---|---|
| A3 | 20 nM |

The binding data shows the compounds described above have nM affinity for the A3 receptor and are highly selective for the A3 receptor. Such A3 selectivity minimizes or eliminates side effects associated with other adenosine receptor subtypes such as cardiovascular effects, e.g., hypotension, bradycardia, arrthymias, and CNS effects, e.g., sedation, locomotion.

Specific inhibition of adenosine induced vascular constriction

The vasoconstrictor action of adenosine in hamster cheek pouch arterioles is described here, and blockade of this response by A3 adenosine receptor antagonists is demonstrated. Adenosine, inosine, cromolyn, compound 48/80, methylene blue, acetylcholine, and components for saline solutions used to bathe arterioles are obtained from

Sigma. 8(p-sulphophenyl)theo-phylline was obtained from Research Biochemicals, Inc. (Natick, MA).

Arterioles (luminal diameter approximately 60 μm) are dissected from male Golden hamster cheek pouches, transferred to a 37°C tissue chamber, and cannulated at both ends (see Duling *et al.*, *Am. J. Physiol.*, (241) (*Heart Circ. Physiol.*, (10) pp. H108-H116 (1981); Duling *et al.*, *Microcirculatory Technology*, edited by C.H. Baker and W.G. Nastuk, Orlando Academic Press, pp. 265-280 (1986)). Changes in luminal diameter in response to abluminal delivery of adenosine ($10^{-8}$ M to $10^{-4}$ M) are measured using videotaped microscopic observation and video calipers with continuous output, to generate cumulative concentration-response curves. These curves are discovered to be biphasic: $10^{-6}$ M adenosine elicited an intense, transient constriction and higher concentrations induced dilator responses. Pretreatment (100 μM) with 8(p-sulfophenyl) theophylline, SPT, a nonspecific adenosine receptor antagonist, inhibited the dilator responses but did not alter the constriction. Without more, this result is consistent with the interpretation that the constrictor response is not mediated through an adenosine A2 receptor.

The constrictor response is assymetrical and focal in nature, such that it was initiated at discrete points and subsequently spread along the entire vessel, suggesting discrete sites of action of adenosine. Examination of the abluminal surface of the vessel after staining with methylene blue reveal large numbers of mast cells closely associated with the vessel wall. Following exposure to adenosine, mast cells are found to be degranulated, suggesting the involvement of mast cell granule contents in the constrictor response. This finding is consistent with reports that adenosine potentiates the release of granule contents from mast cells isolated from rat peritoneum, and that mast cell degranulation causes constriction in some vascular beds resulting in C5a-induced myocardial ischemia, mast cell-dependent inflammation, brain arteriole diameter constriction, and the release of allergic mediators.

Following the above procedure, an antagonist of the formula described above is used to treat tissue, and found to inhibit constriction.

Reduction of myocardial infarct size in anesthetized dogs

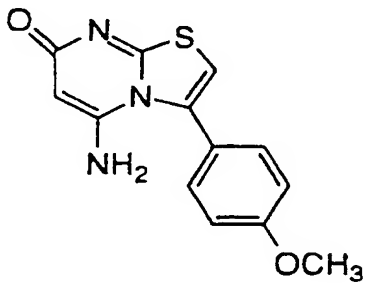
The effect of 3-(4-methoxyphenyl)-5-amino-7-oxo-thiazolo[3,2]-pyrimidine (1-1) on myocardial infarct size is compared to a vehicle-treated control group in barbital-anesthetized dogs subjected to 90 minutes of left anterior descending coronary artery occlusion followed by 3 hours of reperfusion. Vehicle (5 μM NaOH in isotonic saline) or antagonist are infused at a rate of 1 ml/min directly into the left anterior descending coronary artery distal to the occlusion site beginning 10 minutes before occlusion and are continued throughout the entire ischemic period. The myocardial region at risk and infarct size are determined by the triphenyltetrazolium histochemical technique and regional myocardial blood flow by radioactive microspheres. Coronary sinus LDH activity and histamine concentrations were measured at various times throughout the experiments and myeloperoxidase activity was determined at the conclusion of the experiments as an index of neutrophil infiltration into the ischemic-reperfused mycocardium. Ten dogs were included in this preliminary study, 4 in the vehicle-treated group and 6 in the antagonist treated group.

In all animals, arrhythmias are encountered during occlusion and reperfusion and some dogs from each group progress to ventricular fibrillation requiring cardioversion. Hemodynamics and regional myocardial blood flow in the non-ischemic left circumflex coronary artery region are not different between groups at baseline or during occlusion although during reperfusion dP/dt is significantly improved and left anterior descending coronary artery blood flow was significantly decreased in antagonist treated dogs. In the ischemic-reperfused region, collateral blood flow during the occlusion period (the major determinant of ultimate infarct size) was slightly greater in antagonist-treated dogs, particularly in the subepicardial region. In addition, coronary sinus LDH activity and histamine concentrations are reduced in the antagonist-treated group during reperfusion. Finally, myeloperoxidase activity activity is not different between the two groups although there is a tendency for reduced activity in drug-treated animals in infarcted tissue.

1-1 effectively reduces myocardial infarct size in anesthetized dogs by a direct cardioprotective action.

While the foregoing specification teaches the principles of the present invention, with examples provided for the purpose of illustration, it will be understood that the practice of the invention encompasses all of the usual variations, adaptations, modifications, as come within the scope of the following claims and its equivalents.

Formulations with the active A3 antagonist




1-1

were prepared.

## EXAMPLE 2

Asthma Treatment

The A3 adenosine receptor antagonists can also be administered by inhalation. Commercially available nebulizers for liquid formulations, including jet nebulizers and ultrasonic nebulizers are useful for such administration. Liquid formulations can be directly nebulized and lyophilized powder can be nebulized after reconstitution.

For administration by inhalation, the antagonists are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or nebulizers. The compounds may also be delivered as powders which may be formulated and the powder composition may be inhaled with the aid of an insufflation powder inhaler device.

An exemplary delivery system for inhalation is a metered dose inhalation (MDI) aerosol, which may be formulated as a suspension or solution of an A3 adenosine receptor antagonist in suitable propellants, such as fluorocarbons or hydrocarbons.

| Aerosol | Per canister |
|---|---|
| Compound 1-1 | 24 mg |
| Lecithin, NF Liquid Concentrate | 1.2 mg |
| Trichlorofluoromethane, NF | 4.025 g |
| Dichlorodifluoromethane, NF | 12.15 g |

The aerosol formulation is prepared according to conventional procedures and administered to a patient experiencing an asthmatic episode.

## EXAMPLE 3

Treatment of allergies caused by airborne allergens

Tablets and capsules represent a dosage form suitable for patients suffering from allergies caused by airborne allergens. To make a tablet, compound 1-1, in a free-flowing form such as powder or granules, is introduced to a suitable machine, optionally mixed with a binder, lubricant, inert diluent, surface active or dispersing agent. Desirably, each tablet contains from about 2.5 mg to about 500 mg of the antagonist and each cachet or capsule contains from about 2.5 to about 500 mg of the antagonist.

The following are representative tablet and capsule formulations:

| Tablet | mg/tablet |
|---|---|
| Compound 1-1 | 25 |
| Microcrystalline Cellulose | 415 |
| Pregelatinized Starch | 43.5 |
| Magnesium Stearate | 2.5 |

| Capsule | mg/capsule |
|---|---|
| Compound 1-1 | 25 |
| Lactose Powder | 573.5 |
| Magnesium Stearate | 1.5 |

A patient suffering from allergies caused by airborne allergens takes 1 or 4 tablets or capsules each day to alleviate and/or prevent allergy symptoms

## EXAMPLE 4

Psoriasis treatment

Included within the invention are preparations for topical application to the skin whereby the A3 adenosine receptor antagonists are effective in the treatment and control of skin diseases characterized by rapid rates of cell proliferation and/or abnormal cell proliferation, e.g., psoriasis.

In a preferred method of treating hyperproliferative skin diseases, a pharmaceutical formulation comprising an A3 adenosine receptor antagonist (usually in concentrations in the range of from about 0.001 percent to about 10 percent, preferably from about 0.1 percent to about 5 percent), together with a non-toxic, pharmaceutically acceptable topical carrier, is applied several times daily to the affected skin until the condition is improved. Topical applications may then be continued at less frequent intervals (e.g., once a day) to control mitosis in order to prevent return of severe disease conditions.

The topical pharmaceutical compositions according to the invention may also include one or more preservatives or bacteriostatic agents, e.g., methyl hydroxybenzoate, propyl hydroxybenzoate, chlorocresol, benzalkonium chlorides, etc.

| Ointment | mg/g |
|---|---|
| Compound 1-1 | 1.0-20.0 |
| Benzyl Alcohol, NF | 20.0 |
| Mineral Oil, USP | 50.0 |
| White Petrolatum, USP to make | 1.0 g |

A patient suffering from psoriasis administers the ointment to affected areas several times each day.

## EXAMPLE 5

Recanulation treatment

Compound 1-1 is used in adjunct therapy with recanulation such as angioplasty and thrombolytic agents for the treatment of reperfusion injury.

An i.v. formulation such as

| | |
|---|---|
| Compound 1-1 | 0.5-10.0mg |
| Sodium Citrate | 5-50mg |
| Citric Acid | 1-15mg |
| Sodium Chloride | 1-8mg |
| Water for Injection (USP) | q.s. to 1 L |

is administered to a patient undergoing angioplasty or thrombolytic therapy. For preparation of the formulation, compound 1-1 is dissolved at room temperature in a previously prepared solution of sodium chloride, citric acid, and sodium citrate in Water for Injection (USP, see page 1636 of United States Pharmacopeia/National Formulary for 1995, published by United States Pharmacopeial Convention, Inc., Rockville, Maryland, copyright 1994)

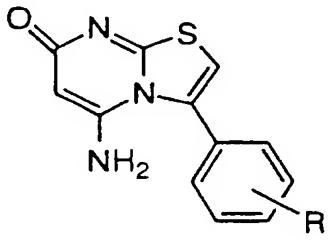
Following i.v. treatment, oral compositions of the compound, such as

| Tablet | mg/tablet |
|---|---|
| Compound 1-1 | 25 |
| Microcrystalline Cellulose | 415 |
| Pregelatinized Starch | 43.5 |
| Magnesium Stearate | 2.5 |

are administered for two weeks.

WHAT IS CLAIMED IS:

1. A method for inhibiting activation of the human A3 adenosine receptor by adenosine, by treating a patient in need thereof with a compound of the formula



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and pharmaceutically acceptable salts thereof, wherein R is selected from the group consisting of

hydrogen,
chlorine,
bromine,
fluorine,
iodine,
hydroxyl,
$C_{1-4}$ alkyl,
$C_{1-4}$ alkyloxy, and
$C_{1-4}$ alkylcarboxy.

2. A method of Claim 1, wherein R is hydrogen, $C_{1-4}$ alkyl, or $C_{1-4}$ alkyloxy.

3. A method of Claim 2, wherein R is $-OCH_3$.

4. A method for achieving blockade of the vasoconstrictive response by treating a patient in need thereof with a compound of formula I of Claim 1.

5. A method for treating or preventing myocardial ischemia, inflammation, brain arteriole diameter constriction, and the release of allergic mediators by treating a patient in need thereof with a compound of formula I of Claim 1.

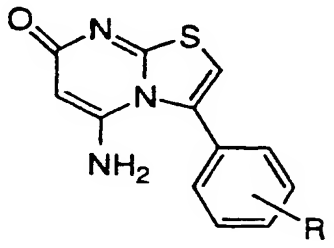
6. A method for treating or preventing asthma, myocardial reperfusion injury, rhinitis, poison ivy induced responses, urticaria, scleroderma, arthritis, and inflammatory bowel diseases by treating a patient in need thereof with a compound of formula I of Claim 1.

7. A method for preventing mast cell degranulation in a human by treating a patient in need thereof with a compound of formula I of Claim 1.

8. A method for achieving blockade of the vasoconstrictive response by treating a patient in need thereof with a compound of formula I of Claim 1, wherein R is -$OCH_3$.

9. A method for treating or preventing asthma, myocardial reperfusion injury, rhinitis, poison ivy induced responses, urticaria, scleroderma, arthritis, and inflammatory bowel diseases by treating a patient in need thereof with a compound of formula I of Claim 1, wherein R is -$OCH_3$.

10. A compound having the formula




I

and pharmaceutically acceptable salts thereof, wherein R is selected from the group consisting of

fluorine,
iodine,
$C_{2-4}$ alkyl,
$C_{3-4}$ alkyloxy, and
$C_{1-4}$ alkylcarboxy.

11. The use of a compound of Claim 10, or a pharmaceutically acceptable salt thereof, in the manufacture of a medicament for treating or preventing asthma, myocardial reperfusion injury, rhinitis, poison ivy induced responses, urticaria, scleroderma, arthritis, and inflammatory bowel diseases in a mammal.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/03648

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : C07D 295/02; A61K 31/505
US CL :544/278; 514/258
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 544/278; 514/258

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y -- A | El-KERDAWY, M. M., et al. Synthesis of novel thiazolopyrimidine and thiazolothiatriazine derivatives, Bull. Fac. Pharm. (Cairo Univ.) 1993, Vol. 31, No. 1, pages 67-74, see entire document. | 1-3, 6, 10 ---------- 4-5, 7-9 |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 29 MAY 1997 | 27 JUN 1997 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230 | Authorized officer<br>MATTHEW V. GRUMBLING<br>Telephone No. (703) 308-1235 |

Form PCT/ISA/210 (second sheet)(July 1992)*

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/03648

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 11
because they relate to subject matter not required to be searched by this Authority, namely:

The claim is drawn to a "Use" with no process step recited. Thus the claim is non-statutory.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.